IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of Akihito TSUCHIYA.

Appln. No. 09/731,863 Group Art Unit: 1744

Filed: 12/08/2000 Examiner: BEISENER, WILLIAM H

For: METHOD FOR PURIFICATION TREATMENT OF ENVIRONMENT POLLUTANT

DECLARATION

Honorable Commissioner of Patents and Trademarks
Washington, D.C. 20231
Sir :

I, Akihito TSUCHIYA, hereby declare that:

1) I currently reside at 116-1, Chuo 5-chome, Kosei-cho, Koga-gun, Shiga-ken, Japan. I am currently employed by ALLMIGHTY CO., LTD

2) I am a inventor of the instant invention, and

3) The experiments given below were carried out under my general direction and supervision.

Experiment

1. Purpose of the experiment

This experiment aims to examine, in the purification of an environmental pollutant using a microbial treatment agent, how the type of organic polymer used to incorporate microorganisms influences the degradation rate of the environmental pollutant.

2. Method of the experiment

2.1 Preparation of microorganisms

A liquid medium (composition: 0.5 wt.% of yeast extract, 1.0 wt.% of bacto-tryptone and 1.0 wt.% of sodium

chloride) (150 L) was placed in a 300-liter fermenter (MPF-U type 300-liter fermenter; B. E. Marubishi Co., Ltd.), steam-sterilized (121°C, 20 min), and adjusted to 30°C. Then, ***Bacillus subtilis*** isolated from sewage sludge was inoculated into the medium and incubated for 48 hours under aerobic conditions with agitation at 200 rpm.

The culture solution thus obtained was centrifuged at 3,000 rpm to collect the bacterial cells, which were then dried to obtain a dry bacterial cell powder.

2.2 Preparation of microbial treatment agents

<***Bacillus subtilis***-produced levan-containing microbial treatment agent>

Bacillus subtilis(ATC6633) was inoculated into XX L of a liquid medium (containing 0.22 wt.% of ammonium chloride, 0.3 wt.% of yeast extract, 0.3 wt.% of sucrose, 0.35 wt.% of K_2HPO_4 and 0.5 wt.% of NaCl), and aerobically incubated at 30°C for 2 days. The bacterial cells were removed from the culture solution by centrifugation. Thus obtained culture supernatant was adjusted to pH 8.3, and an aqueous methanol solution (containing 96 wt.% of methanol) (30 L) was added to the obtained culture supernatant (15 L) to form a precipitate. The precipitate was collected by centrifugation, washed with washing solution consisting of ethanol, acetone and petroleum ether by weight ratio of 1:1:1, and vacuum-dried, giving a ***Bacillus subtilis***-produced levan powder.

The ***Bacillus subtilis***-produced levan powder (0.1 g) was added to 5 mL of deionized water heated to 20°C to fully swell the powder. To the resulting ***Bacillus subtilis*** -produced levan solution, 1.2 mg of the bacterial cells obtained above was added, followed by stirring to homogeneity. The bacterial cell/***Bacillus subtilis*** -produced levan suspension thus obtained was added dropwise to a 0.1M $CaCl_2$ solution to form a precipitate, which was collected to obtain a ***Bacillus subtilis*** -produced levan-containing microbial treatment agent. The microbial treatment agent contained 0.2×10^9 cells per gram.

<***Acetobacter bacteria***-produced levan-containing microbial treatment agent>

A ***Acetobacter bacteria***-produced levan-containing microbial treatment agent was prepared by following the procedure for preparing the ***Bacillus subtilis***-produced levan-containing microbial treatment agent, except for for using ***Acetobacter*** (IFO 15606) in place of ***Bacillus subtilis***. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<***Pseudomonas bacteria***-produced levan-containing microbial treatment agent>

A ***Pseudomonas bacteria***-produced levan-containing microbial treatment agent was prepared by following the procedure for preparing the ***Bacillus subtilis***-produced levan-containing microbial treatment agent, except for using ***Pseudomonas*** (NCIMB 9056) in place of ***Bacillus subtilis***. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<β-2,6 fructan-containing microbial treatment agent>

A β-2,6 fructan -containing microbial treatment agent was prepared by following the procedure for preparing the ***Bacillus subtilis***-produced levan-containing microbial treatment agent, except for using ***Enwinia*** (NCIMB 101206) in place of ***Bacillus subtilis***. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<Poly-leucine-containing microbial treatment agent>

Poly-L-leucine (molecular weight of 100,000 to 150,000; Wako Pure Chemical Industries, Ltd.) (5.5 g), acetic acid (0.03 g), and Triton X-100 (Wako Pure Chemical Industries, Ltd.) (0.2 g) were added to 10 mL of deionized water heated to 20°C to prepare a polyamino acid solution. The bacterial cells prepared above (1.2 mg) were added to the poly-L-leucine

solution, followed by stirring at 37°C for 25 minutes. Then, acetone (4 mL) was added to form a precipitate, which was collected by centrifugation to obtain a poly-L-leucine-containing microbial treatment agent. The microbial treatment agent contained 0.2×10^9 cells per gram.

<Poly-alanine-containing microbial treatment agent>

A poly-DL- alanine-containing microbial treatment agent was prepared by following the procedure for preparing the poly-leucine-containing microbial treatment agent, except for using poly-DL- alanine (molecular weight of 1000 to 5,000; Sigma) in place of poly-L-leicune. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<Poly-arginine-containing microbial treatment agent>

A poly-L-arginine-containing microbial treatment agent was prepared by following the procedure for preparing the poly-leucine-containing microbial treatment agent, except for using poly-L-arginine (molecular weight of 15,000 to 30,000; Sigma) in place of poly-L-leicune. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<Poly-lysine hydrobromide-containing microbial treatment agent>

A poly-L-lysine hydrobromide-containing microbial treatment agent was prepared by following the procedure for preparing the poly-leucine-containing microbial treatment agent, except for using poly-L-lysine hydrobromide (molecular weight of 15,000 to 30,000; Wako Pure Chemical Industries, Ltd.) in place of poly-L-leicune. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

<Poly-benzyl-L-aspartate-containing microbial treatment agent>

A poly-benzyl-L-aspartate-containing microbial treatment agent was prepared by following the procedure for

preparing the poly-leucine-containing microbial treatment agent, except for using poly-benzyl-L-aspartate (molecular weight of 15,000 to 50,000; Sigma) in place of poly-L-leicune. The obtained microbial treatment agent contained 0.2×10^9 cells per gram.

2.3 Preparation of synthetic sewage

4-Octylphenol was added as an environmental pollutant, at a concentration of 150 ppm, to an aqueous solution of the following composition (COD: 102 ppm, total nitrogen content: 32 ppm, total phosphorus content: 3.5 ppm) to prepare a synthetic sewage.

Glucose	0.5 g
K_2HPO_4	0.004342 g
KH_2PO_4	0.0017 g
$Na_2HPO_4 \cdot 12H_2O$	0.00892 g
NH_4Cl	0.04674 g
$MgSO_4 \cdot 7H_2O$	0.0225 g
$FeCl \cdot 6H_2O$	0.00025 g
$CaCl_2$	0.0275 g
Polypeptone	0.15 g
Deionized water	Balance
Total	1 L (pH 7.0)

2.4 Degradation test of the environmental pollutant

Each of the microbial treatment agents prepared above was added, at a concentration of 500 ppm, to 20 mL of the synthetic sewage, and incubated at 30°C for 1 week with shaking. After incubation, 10mL of supernatant was drawn from the synthetic sewage after the degradation treatment. To the remainder of the synthetic sewage after the degradation treatment, 10 mL of untreated synthetic sewage was added, and incubation was carried out under the same conditions as above. Such operation ware continued for 5 weeks (total 5 times).

3. Result of the experiment

Table Z shows the remaining proportion (%) of 4-octylphenol after the treatment with each microbial treatment agent, relative to the initial 4-octylphenol concentration. Table A also shows the proportion in case of using alginic acid, carrageenan, or chitosan containing microbial treatment agent, which was disclosed in my declaration submitted on March 17, 2003.

Table Z

	4-Octylphenol remaining proportion (%)		
	After 1 week	After 3 weeks	After 5 weeks
Bacillus subtilis-produced levan	99.8	97.1	86.2
Acetobacter bacteria-produced levan	99.1	86.7	80.6
Pseudomonas bacteria-produced levan	99.6	96.1	84.3
β2,6 fructan	98.3	97.5	86.2
Poly-leucine	99.0	90.8	76.4
Poly-alanine	100	99.2	87.1
Poly-arginine	99.9	96.6	90.1
Poly-lysine hydrobromide	99.1	90.3	88.9
Poly-benzyl-L-aspartate	100	97.4	90.2
Alginic acid	99.8	100.8	100.2
Carrageenan	99.8	101.2	100.0
Chitosan	99.9	101.0	102.0

4. Consideration

The results shown in Table Z confirm that a microbial treatment agent prepared using the specific microorganism-produced polymer (levan produced by ***Bacillus***,

Acetobacter or Pseudomonus; β 2,6 fructan; and various polyamino acids)can effectively degrade 4-octylphenol. In contrast, a microbial treatment agent prepared using alginic acid, carrageenan, or chitosan cannot degrade 4-octylphenol. . Thus, it is presumed that, owing to the effects of the specific microorganism-produced polymer, the microorganisms retain their biological activity necessary for degrading 4-octylphenol.

The test results reveal that the use of the specific microorganism-produced polymers for incorporating microorganisms enables more effective degradation of an environmental pollutant.

I, the undersigned, declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 2003.10.17 Akihito Tachiyama